Prediction of serum IgG concentration by indirect techniques with adjustment for age and clinical and laboratory covariates in critically ill newborn calves

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Abstract

The objective of this study was to develop prediction models for the serum IgG concentration in critically ill calves based on indirect assays and to assess if the predictive ability of the models could be improved by inclusion of age, clinical covariates, and/or laboratory covariates. Seventy-eight critically ill calves between 1 and 13 days old were selected from 1 farm. Statistical models to predict IgG concentration from the results of the radial immunodiffusion test, the gold standard, were built as a function of indirect assays of serum and plasma protein concentrations, zinc sulfate ($ZnSO_4$) turbidity and transmittance, and serum γ-glutamyl transferase (GGT) activity. For each assay 4 models were built: without covariates, with age, with age and clinical covariates (infection and dehydration status), and with age and laboratory covariates (fibrinogen concentration and packed cell volume). For the protein models, dehydration status (clinical model) and fibrinogen concentration (laboratory model) were selected for inclusion owing to their statistical significance. These variables increased the coefficient of determination (R^2) of the models by ≥ 7% but did not significantly improve the sensitivity or specificity of the models to predict passive transfer with a cutoff IgG concentration of 1000 mg/dL. For the GGT assay, including age as a covariate increased the R^2 of the model by 3%. For the $ZnSO_4$ turbidity test, none of the covariates were statistically significant. Overall, the R^2 of the models ranged from 34% to 62%. This study has provided insight into the importance of adjusting for covariates when using indirect assays to predict IgG concentration in critically ill calves. Results also indicate that $ZnSO_4$ transmittance and turbidity assays could be used advantageously in a field setting.

Résumé

L'objectif de cette étude était de développer un modèle de prédiction de la concentration sérique des IgG chez des veaux malades à partir de techniques indirectes, et d'évaluer si la capacité de prédiction du modèle peut être améliorée par l'inclusion de l'âge et de certains paramètres clinique et de laboratoire. Soixante-dix-huit veaux gravement malades âgés entre 1 et 13 jours et élevés sur une même ferme ont été sélectionnés. Des modèles statistiques pour prédire la concentration sérique des IgG mesurée par immunodiffusion radiale (épreuve de référence) ont été construits à partir de mesures indirectes (concentration des protéines sériques, concentration des protéines plasmatiques, turbidité au sulfate de zinc, transmittance au sulfate de zinc, concentration sérique de la GGT (γ-glutamyl transférase). Pour chacune des techniques indirectes, 4 modèles ont été construits : sans covariable, avec l'âge seulement, avec l'âge et des paramètres cliniques (présence d'un foyer d'infection et état d'hydratation), et avec l'âge et les paramètres de laboratoires (concentration plasmatique en fibrinogène, hématocrite). Pour les modèles incluant les protéines sériques et plasmatiques, l'état d'hydratation et la concentration plasmatique en fibrinogène ont été retenus (statistiquement significatif). L'inclusion de ces variables augmentait la valeur du R^2 *des deux modèles de ≥ 7 %, mais n'avait pas d'impact significatif sur la sensibilité ou spécificité des modèles pour prédire le transfert de l'immunité passive (utilisant une valeur seuil de 1000 mg/dL). Pour le modèle GGT, l'âge a été retenu comme covariable et son inclusion augmentait le* R^2 *de 3 %. Pour les modèles de sulfate de zinc, aucune covariable n'était statistiquement significative. La valeur du* R^2 *des différents modèles construits variait de 34 % à 62 %. Les résultats de cette étude soutiennent l'importance d'inclure certains autres paramètres pour prédire le succès du transfert de l'immunité passive à partir de techniques indirectes dans une population de veaux gravement malades. Ils indiquent également que les techniques de turbidité et de transmittance au sulfate de zinc sont appropriées pour une utilisation à la ferme.*

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Introduction

Partial or complete failure of passive transfer (FPT) of immunoglobulins to suckling calves is a significant problem, increasing the risk of both illness and death (1–3). It is assessed by measuring the serum IgG concentration, ideally within 24 to 48 h after birth. Radial immunodiffusion (RID) is the only test that directly measures this concentration, and it is considered the gold standard.

In practice, evaluation of the serum IgG concentration is often done in older calves with neonatal disease. The level is rarely measured directly. Instead, less expensive and more rapid tests measuring the concentrations of total globulins or other proteins known to be correlated with the IgG concentration (3,4) are generally used. Available tests include measurements of the serum concentration of total solids by refractometry, sodium sulfate turbidity, zinc sulfate ($ZnSO_4$) turbidity, serum γ-glutamyl transferase (GGT) activity, and whole blood glutaraldehyde gelation.

In previous studies the interpretation and validation of the results of various indirect assays for the serum IgG concentration were generally based on defining a cutoff value for predicting the failure or adequacy of passive transfer (5,6). However, in a clinical setting such an approach does not allow for critical appraisal of the level of IgG passive transfer, which may directly influence decision-making in regard to treatment and prognosis. Moreover, the impact of different clinical parameters, such as dehydration and sepsis, on the association between a particular indirect assay and the gold standard was not taken into account in previous studies and warrants further investigation.

This study proposed prediction models based on various indirect assays for estimating the serum IgG concentration in ill calves and compared the predictive ability of the models with and without the inclusion of age, clinical covariates, and laboratory covariates. The null hypothesis was that these covariates do not have an impact on the association between various indirect assays and the serum IgG concentration.

Materials and methods

The data used in this study originated from an observational study based on a convenience sample. From a large calf-rearing farm in the San Joaquin Valley of California, we selected 78 critically ill male Holstein calves between 1 and 13 d of age with a total clinical score of 5.5 or higher. This score is based on evaluation of feces, hydration status, calf behavior, umbilicus, and scleral vessel characteristics (7). There was no information available on colostrum feeding since the animals arrived on the premises at approximately 1 d of age. At the time of selection the calves were clinically evaluated, age was noted, and blood samples were collected by a previously described technique (8).

Clinical evaluation

The presence of infection and hydration status were recorded according to a previously described protocol (7). Infection was considered present if at least 1 of the following was noted: a positive blood culture, visual detection of an infection site (e.g., septic joint, hypopyon, mucopurulent discharge, or abscess), or an umbilical score ≥ 2. Calves presenting obvious signs of dehydration (such as sunken eyes) were considered dehydrated.

Table I. Descriptive statistics for the variables used in models to predict the serum IgG concentration in 78 critically ill calves

Continuous variables	Unit	Range	Median
Assay			
Serum protein concentration	g/L	36–81	46
Plasma protein concentration	g/L	43–90	56
$ZnSO_4$ transmittance	%	0–64	32
Serum GGT activity	IU/L	8–586	19
Demographic covariate			
Age	Days	1–13	5
Laboratory covariate			
Packed cell volume	%	21–54	35
Fibrinogen concentration	g/L	3–16	7
Categorical covariates	**Category**		***n***
Assay			
$ZnSO_4$ turbidity	Total failure		39
	Partial failure		26
	Adequate protection		13
Clinical covariate			
Infection	Yes		16
	No		62
Dehydration	Yes		19
	No		59

$ZnSO_4$ — zinc sulfate; GGT — γ-glutamyl transferase.

Laboratory evaluation

Within 8 h of collection, blood samples were centrifuged and analyzed. Packed cell volume (PCV) was estimated in microcapillary tubes, fibrinogen concentration was evaluated by the heat precipitation method, and serum and plasma protein concentrations were determined with a refractometer. For all other analyses, serum was frozen at −20°C for a maximum of 6 mo. The concentration of IgG was evaluated with an RID kit (Immunoglobulins Bovine IgG Kit; ICN Biomedicals, Costa Mesa, California, USA) according to manufacturer specifications. The RID plates were read with an electronic plate reader (Binding Site, San Diego, California, USA). Serum GGT activity was determined with the Synchron CX5 device (Beckman Coulter, Brea, California, USA). Zinc sulfate turbidity and transmittance were evaluated with a $ZnSO_4$ heptahydrate solution prepared by adding 250 mg of $ZnSO_4.7H_2O$ to 1 L of distilled water. Approximately 4 mL of the solution was put in glass vacuum tubes 16 mm in diameter, with caution taken to maintain negative pressure, and 0.1 mL of serum was added. After gentle mixing, the tubes were incubated for 1 h at room temperature. Zinc sulfate turbidity was then evaluated semiquantitatively (for total failure, partial failure, or adequate protection) by placing newspaper behind the tubes: a tube through which letters could be easily distinguished was categorized as showing total failure, a tube through which letters were illegible but detectable as shadows was designated as showing partial failure, and a totally opaque tube was categorized as showing adequate

Table II. Estimated coefficient of regression (β) with standard error (SE) and *P*-value for various linear regression models to predict the log-transformed serum IgG concentration in the calves[a]

Model and variable	β	SE	*P*-value
Serum protein			
No covariate (R^2 = 41%)			
Intercept	−0.450	0.365	0.22
Serum protein level	0.054	0.007	< 0.001
Age and clinical covariates (R^2 = 49%)			
Intercept	−0.679	0.350	0.06
Serum protein level	0.061	0.007	< 0.001
Dehydration	−0.448	0.133	< 0.01
Age and laboratory covariates (R^2 = 48%)			
Intercept	−0.138	0.360	0.70
Serum protein level	0.057	0.007	< 0.001
Fibrinogen concentration	−0.062	0.020	< 0.01
Plasma protein			
No covariate (R^2 = 34%)			
Intercept	−0.502	0.428	0.24
Plasma protein level	0.046	0.007	< 0.001
Age and clinical covariates (R^2 = 42%)			
Intercept	−0.803	0.416	0.06
Plasma protein level	0.053	0.007	< 0.001
Dehydration	−0.451	0.143	< 0.01
Age and laboratory covariates (R^2 = 50%)			
Intercept	−0.431	0.376	0.26
Plasma protein level	0.058	0.007	< 0.001
Fibrinogen concentration	−0.100	0.020	< 0.001
GGT			
No covariate (R^2 = 47%)			
Intercept	0.578	0.202	< 0.01
GGT activity (log-transformed)	1.103	0.135	< 0.001
Age (R^2 = 50%)			
Intercept	0.313	0.233	0.18
GGT activity (log-transformed)	1.175	0.136	< 0.001
Age	0.031	0.014	0.04
$ZnSO_4$ transmittance			
No covariate (R^2 = 62%)			
Intercept	3.210	0.104	< 0.001
Transmittance	−0.033	0.003	< 0.001
$ZnSO_4$ turbidity			
No covariate (R^2 = 60%)			
Intercept	1.746	0.068	< 0.001
Adequate protection	1.433	0.136	< 0.001
Partial failure	0.557	0.108	< 0.001

[a] The log (IgG) can be predicted using the following equations:

$$\log(\text{IgG}) = \beta\text{Intercept} + \Sigma\beta\times$$

Where × = the value of the variable for the individual and β is the estimated regression coefficient for this variable.

$$\text{Predicted log(IgG)} = \beta_{\text{Intercept}} + [\beta_{\text{Plasma proteins}} \times \text{Plasma proteins (g/L)}] + [\beta_{\text{Fibrinogen}} \times \text{Fibrinogen (g/L)}]$$

$$\text{Predicted log(IgG)} = -0.431 + [0.058 \times 60] + [-0.100 \times 7]$$

$$\text{Predicted log(IgG)} = 2.349$$

Figure 1. Equation used to predict the log serum IgG concentration from the plasma protein model developed in this study with age and laboratory covariates. This model included the intercept and the plasma protein and fibrinogen concentrations as variables according to the model selection building procedure.

protection. Zinc sulfate transmittance was obtained by reading the optical density of each tube with a spectrophotometer (Coleman Spec 20; PerkinElmer, Downers Grove, Illinois, USA) at 485 nm. All $ZnSO_4$ assays were performed the same day by the same investigator on the same $ZnSO_4.7H_2O$ preparation.

Statistical analysis

For each of the 5 assays, 4 linear regression models were built with the logarithmic transformation (base 10) of the serum IgG concentration measured by RID as the dependent variable (9). The assay results were put in the models in their original scales, apart from the GGT results, which were log-transformed (base 10). The log transformations were used to improve the normality and linearity of the residuals. Full main-effect models were built first. Of the 4 models, the 1st included only the assay, the 2nd also included age, and the 3rd and 4th models also included age and either clinical covariates (dehydration and infection) or laboratory covariates (PCV and fibrinogen concentration), respectively. A backward-elimination procedure based on the F-test with $P > 0.05$ as the criterion of elimination was then used. Variables were removed only if removal did not change the estimated coefficient of the assay by more than 20%. First-order interactions between assay and covariates were tested 1 at a time with the resulting models and were selected for inclusion in the final model if their *P*-value was less than 0.05 (F-test). For each final model the coefficient of determination (R^2) was computed. Normal distribution of the residuals was visually assessed on a normal-probability plot. Outliers were detected visually as well as by using studentized residuals and the difference in fitted values (DFFITs) after deletion of a case. Studentized residuals were plotted against the predicted value to evaluate homoscedasticity and against each continuous predictor variable to evaluate the linearity of the regression function. When an outlier was identified, the model was refitted to exclude the outlier, and changes in the coefficient or *P*-value were studied. All statistical analyses were performed with the PROC GLM procedure in SAS 9.1 (SAS Institute, Cary, North Carolina, USA).

The ability of each final model to predict passive transfer status was computed. The RID test result was used as the gold standard: calves were classified as having FPT if the serum IgG concentration was less than 1000 mg/dL and otherwise were considered to have adequate passive transfer (APT). The sensitivity of the various models was estimated as the percentage of calves with predicted FPT (gauged by model predictions transformed from a logarithmic scale to the original scale) among the calves with FPT. Specificity was estimated as the percentage of calves with predicted APT among the calves with APT. Confidence intervals (95%) for the sensitivity and specificity estimates were calculated with the PROC FREQ procedure in SAS 9.1 with the use of exact computations for binomial distributions. For each assay, McNemar tests with exact computations were performed to determine if the sensitivity and specificity differed significantly ($\alpha = 0.05$) between each 2×2 combination of the 4 models. The positive predictive value for each model was estimated as the probability (%) of FPT among the calves with predicted FPT. Similarly, the negative predictive value for each model was estimated as the probability (%) of APT among the calves with predicted APT. These predictive values were calculated with the assumption of the same proportion of FPT in the population as observed in the study sample.

Results

In the 78 calves included in the study, the serum IgG concentration ranged from 5 to 6980 (median 135) mg/dL. With a cut-off value of 1000 mg/dL the proportion of calves with FPT was 86%. Descriptive statistics for the assay results and covariates are presented in Table I.

The final linear regression models are presented in Table II. Only models with covariates selected for inclusion are presented. For the serum protein, plasma protein, and GGT assays, at least 1 covariate was kept in the final model owing to its statistical significance ($P < 0.05$). For the 2 assays based on $ZnSO_4$, none of the tested covariates was significantly associated with the log-transformed serum concentration of IgG.

During the model building, no evidence of confounding was detected, and no interactions were statistically significant (all $P \geq 0.10$). Visual analysis of the residuals did not reveal any important departure from normality, homoscedasticity, or linearity of the regression function. No outlier was detected visually or according to studentized residuals (all ≤ 2.8) or DFFITs (all ≤ 0.64) except for a relatively high studentized residual value (3.15) in a single model in 1 calf. Because exclusion of this value did not influence model selection and had only a minor impact on coefficient estimates (< 15%), it was kept for analysis.

The estimated coefficients of regression were used to predict the log IgG serum concentration for each calf with each model. As an example, the equation presented in Figure 1 was used to predict the log IgG concentration from the plasma protein model with age and laboratory covariates in a calf with a plasma protein concentration of 60 g/L and a fibrinogen concentration of 7 g/L. The predicted log IgG value was then back-transformed on its original scale (i.e., $10^{2.349} = 223$ mg/dL) (Figure 1).

The sensitivity, specificity, and predictive values of the various models are presented in Table III. No statistically significant differ-

Table III. Sensitivity, specificity, positive and negative predictive values of the various models in predicting failure of passive transfer (FPT) of immunoglobulins to the calves, with a cut-off for the IgG concentration of < 1000 mg/dL and radial immunodiffusion (RID) as the gold standard

Model and covariates included	Sensitivity (95% confidence interval)[a]	Specificity (95% confidence interval)[b]	Positive predictive value[c]	Negative predictive value[d]
Serum protein				
None	100 (95, 100)	18 (2, 52)	88 (67/76)	100 (2/2)
Dehydration	100 (95, 100)	27 (6, 61)	89 (67/75)	100 (3/3)
Fibrinogen level	100 (95, 100)	45 (17, 77)	92 (67/73)	100 (5/5)
Plasma protein				
None	100 (95, 100)	27 (6, 61)	89 (67/75)	100 (3/3)
Dehydration	100 (95, 100)	27 (6, 61)	89 (67/75)	100 (3/3)
Fibrinogen level	100 (95, 100)	36 (11, 69)	91 (67/74)	100 (4/4)
GGT				
None	97 (90, 100)	27 (6, 61)	89 (65/73)	60 (3/5)
Age	99 (92, 100)	27 (6, 61)	89 (66/74)	75 (3/4)
$ZnSO_4$ transmittance				
None	99 (92, 100)	73 (39, 94)	96 (66/69)	89 (8/9)
$ZnSO_4$ turbidity				
None	94 (85, 98)	82 (48, 98)	97 (63/65)	69 (9/13)

[a] Percentage of calves with FPT as predicted by the model among the 67 calves with FPT according to RID.

[b] Percentage of calves with adequate passive transfer (APT) as predicted by the model among the 11 calves with APT according to RID.

[c] Numbers in parenthesis represent the number of calves with FPT as predicted by the model over the number of calves with FPT according to RID, based on an 86% prevalence of FPT in the sample.

[d] Numbers in parenthesis represent the number of calves with APT as predicted by the model over the number of calves with APT according to RID, based on an 86% prevalence of FPT in the sample.

ence in sensitivity or specificity was detected between the 4 models of each assay (all $P > 0.25$ by the McNemar exact test).

Discussion

In this study, FPT was common, affecting 86% of the study population. Although this proportion is not representative of that in the normal population of dairy calves, it may reflect the occurrence in sick calves presented to veterinarians. All the animals were male Holstein calves fed and housed in an identical manner and sampled over a short period (within 5 d), and all the blood samples were stored and analyzed in an identical manner. This protocol likely minimized the number of potential confounding covariates. The solution used for the evaluation of $ZnSO_4$ turbidity and transmittance was prepared with a 250-mg/L solution rather than the typical 208-mg/L solution (4). In our clinical experience, adding a 3rd category (for partial failure) instead of relying on the traditional 2 categories makes readings easier; it is also likely to provide more information about the serum IgG concentration.

Among the clinical covariates, hydration status had a significant effect of similar magnitude in the serum and plasma protein models. Clinical dehydration was associated with a decrease in serum IgG concentration, perhaps because of poor suckling as a common factor. However, this association was unexpected since PCV, considered an indicator of dehydration, was not selected for inclusion in the laboratory models. Further exploration of the data showed that hydration status is a poor predictor of PCV in a simple linear regression model ($R^2 = 3\%$, $P = 0.12$). Thus, other unidentified covariates might play an important role in the observed relationship.

Among the laboratory covariates, fibrinogen concentration was a significant predictor of IgG concentration in the models based on serum and plasma protein concentrations estimated by refractometry. Inclusion of the fibrinogen concentration most likely provided an adjustment for the presence of nonimmunoglobulin proteins, such as acute-phase proteins, in the presence of infection. When fibrinogen concentration was included as a covariate the R^2 increased 7% with the serum model and 16% with the plasma model. This observation supports the importance of such adjustment for IgG prediction. Age had a significant effect in the GGT model. An increase in serum IgG concentration was observed as age increased, as previously reported (10). However, the inclusion of age had a minor impact on the R^2 of the model.

The coefficient of determination for the 10 models ranged from 34% to 62%. Although this indicates that about half of the changes

in IgG concentration could be explained by a particular assay and by the covariates included in the model, it also reminds us that almost half of the variations remain unexplained. Interestingly, the highest R^2 was observed in the model that included $ZnSO_4$ transmittance with no covariate.

The use of mathematical equations to predict IgG concentration on the basis of indirect assays can be useful in a clinical setting, allowing adjustment for covariates and facilitating comparison of the results obtained from various assays. The equations give an estimate on a continuous scale, which is highly informative. The prediction could be done directly by a practitioner using a simple calculator or a program in computerized medical records for a direct result based on input values for various tests. The predicted IgG could then be directly interpreted or classified as adequate or not adequate. Based on a cut-off of 1000 mg/dL, the models developed in this study were very sensitive at predicting FPT, 94% to 100% of the calves with an IgG concentration $<$ 1000 mg/dL being correctly classified. However, the specificity of the models to predict APT was highly variable, ranging from 18% to 45% for the serum protein, plasma protein, and GGT models; it was more satisfactory for $ZnSO_4$ transmittance (73%) and turbidity (82%). Adjustment with covariates did not allow a significant increase in the ability of the models to correctly classify passive transfer. However, the evaluation of the models' ability to predict APT was based on a very limited population of calves and thus should be interpreted as preliminary. The high sensitivity of the models combined with the high prevalence of FPT (86%) led to very good predictive values, although the negative predictive values were based on a very small sample. In a clinical setting, predictive values are of great interest because they can be interpreted as the probability that a calf with a predicted condition truly has the condition according to the gold standard. These values are influenced by the prevalence of FPT in the population and should be extrapolated only to similar populations or be recomputed for populations with different expected prevalence rates. However, in a clinical setting the proportion of ill calves with FPT is likely to be high, as was observed in this study.

These results suggest that for some assays the inclusion of clinical or laboratory covariates improves the accuracy of IgG-concentration predictions, particularly when the results are interpreted on a continuous scale. In general, the $ZnSO_4$ transmittance and turbidity assays performed well in predicting the IgG concentration and could be used advantageously in a field setting.

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